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MicroRNA-572 targets CDH1 to promote metastasis of Wilms' tumor

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Abstract. – INTRODUCTION: Wilms' tumor (WT) is the most common childhood tumor, and recent studies have demonstrated that abnormal expression of microRNA (miRNA) plays an important role in the progression of WT. However, the effect of miR-572 on cell metastasis and epithelial-mesenchymal transition (EMT) in WT is unclear.

PATIENTS AND METHODS: The alternation of miR-572 and cadherin 1 (CDH1) expression was assessed by quantitative Real-time polymerase chain reaction (qRT-PCR). Transwell assay was performed to explore the effects of miR-572 and CDH1 on the metastasis of WT cells. The EMT markers and CDH1 expressions were detected by Western blot. The relationship between miR-572 and CDH1 was verified by dual-luciferase reporter assay.

RESULTS: Upregulation of miR-572 was identified in WT tissues. Increased miR-572 promoted cell metastasis and EMT progress in WT. Moreover, miR-572 directly targeted CDH1 and negatively regulated CDH1 expression in WT tissues. The knockdown of CDH1 showed a promoting effect on cell metastasis and EMT, while overexpression of CDH1 significantly weakened the effect of miR-572.

CONCLUSIONS: MiR-572 targeted CDH1 to promote cell metastasis in WT by suppressing EMT.

Key Words:
Wilms' tumors, miR-572, Metastasis, Epithelial-to-mesenchymal transition, CDH1.

Introduction

Wilms' tumor (WT) is one of the major cancer types in pediatrics and has become a major cause of pediatric-related cancer mortality worldwide[1]. Spreafico et al[2] have shown that the incidence of WT in children has gradually increased, and the mortality rate of WT remains high. Recently, significant progress has been made in the treatment of WT patients with an overall survival rate of over 90%[3]. Despite this, approximate 25% of WT patients cannot be cured depending on existing treatments[4]. Although advances have been made in understanding the pathogenesis of WT, the underlying molecular mechanisms of WT progression still need to be further elucidated. MicroRNAs (miRNAs) has been well known to function as tumor suppressors or oncogenes in the development of various cancer by post-transcriptionally regulation[5]. There are a variety of miRNAs reported to exhibit their function through regulating the expressions of target genes in the progression of WT. For example, miR-613 suppressed cell proliferation, migration and invasion by targeting FRS2 in WT[6]. Jiang et al[7] also reported that miR-1180-5p induced apoptosis of WT cells by targeting p73. Recently, the different effect of miR-572 has caught our attention. Yu et al[8] demonstrated that miR-572 improved early post-operative cognitive dysfunction by downregulating NCAM1. The interaction between miR-572 and PPP2R2C, as well as its carcinogenic effect on proliferation, migration and invasion of nasopharyngeal carcinoma cells, were identified[9]. Similarly, miR-572 prompted the proliferation of human ovarian cancer cells by suppressing PPP2R2C expression[10]. Inversely, downregulation of miR-572 was downregulated in basal cell carcinoma and exerted an inhibitory effect on the development of carcinoma[11]. However, the effect of miR-572 on WT cell metastasis remains largely unknown. By prompting tumor cells to acquire malignant tumor-associated phenotypes, epithelial-mesenchymal transition (EMT) is an important transformation process for malignant tumors[12]. Recently, Terry et al[13] have proposed that EMT is closely associated with tumor invasion and metastasis. Moreover,

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multiple miRNAs have been reported to regulate EMT processes and thus participate in the pathogenesis of cancers. For instance, miR-21 improved the invasion and migration of lung adenocarcinoma cell and EMT through targeting HBP1[14]. The CDH1-encoded E-cadherin is a calcium-dependent cell adhesion protein and belongs to the cadherin family[15]. The CDH1 gene is involved in regulating cell adhesion, migration and proliferation of epithelial cells. The loss of CDH1 leads to an easier cell differentiation, invasion and metastasis[16]. Moreover, the abnormal expression of CDH1 was closely related to the development of gastric cancer, breast cancer, and glioma[17-19]. However, there is almost no study about the effect of CDH1 in metastatic of WT. In the present study, the function of miR-572 was investigated in WT. We initially found that miR-572 abnormally expressed in WT tissues and was inversely related to CDH1 expression. Therefore, we hypothesized that miR-572 might regulate the cell metastasis in WT by modulating CDH1 expression.

Patients and Methods

Patients and Tissue Specimens

A total of 61 tissue samples were obtained from WT patients who received debulking surgery in Weifang People's Hospital. All patients provided written informed consent. WT tissues were stored at -80°C for subsequent experiments. This study was approved by the Weifang People's Hospital Ethics Committee.

Cell Culture and Transfection

The HFWT (RCB; RCB0665) and 17-94 (DSMZ; ACC 741) WT cell lines were used in this study. These cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 5% CO_2 at 37°C. The miR-572 mimic and inhibitor, CDH1 siRNA (si-CDH1) were purchased from Ribobio (Guangzhou, China) and transferred into 17-94 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instruction.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from WT tissues and cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The complementary deoxyribose nucleic acid (cDNA) was reversely transcribed by RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, the qRT-PCR was performed using TaqMan Universal PCR Master Mix Kit (Thermo Fisher Scientific, Waltham, MA, USA) on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of miR-572 and CDH1 were normalized with U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, respectively. The results were quantified with the $2^{-\Delta\Delta ct}$ method. Primer sequences used in this study were as follows: CDH1, F: 5'-CCAGGAACCACTC-3', R: 5'-GCTAGGGATGAAGGA-3'; MiR-572, F: 5'-CCATAGTAAACATCGACTG-3', R: 5'-ACATTGTGTCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

NP-40 lysate (Beyotime, Shanghai, China) was used to extract the protein of 17-94 cells. Proteins were separated through a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocked in 5% non-fat milk, the membranes were then incubated with CDH1, GAPDH and EMT markers (E-cadherin, N-cadherin and Vimentin) primary antibodies overnight at 4°C, and subsequently incubated with the corresponding secondary antibodies. Enhanced chemiluminescence (ECL) Western Blotting Kit (Pierce, Rockford, IL, USA) was then used to detect the antibody binding.

Transwell Assays

The upper chamber surface of the bottom membrane of the transwell chamber was coated with Matrigel (BD, Franklin Lakes, NJ, USA), and the Matrigel was polymerized into a gel at 37°C for 30 min. The 17-94 cell suspension (5×10^5 /mL, 100 μL) was added to the transwell chamber,; next, the medium containing 20% fetal bovine serum (FBS) (600 μL) was added to the lower chamber. After routine incubation for 24 h, the transwell chamber was fixed in methanol for 30 minutes, and stained with 0.1% crystal violet for 20 min. The number of invading cells was observed under a microscope of 400 times. For migration assay, the cells were put in the upper chamber without Matrigel (BD,

Franklin Lakes, NJ, USA). The other steps are the same as the invasion assay.

Dual Luciferase Assay

The wild or mutant 3'-UTR CDH1 vectors (Promega, Madison, WI, USA) were co-transfected into 17-94 cells with miR-572 mimics. After 48 h, 10 μL transfected cells were then measured for luciferase activity through the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

Experimental data were analyzed by Statistical Product and Service Solutions (SPSS) 17.0 and Graphpad Prism 6 (La Jolla, CA, USA). Data are expressed as mean ± SD. The correlation between miR-572 and clinical features of WT patients was analyzed by chi-square test. The overall survival and survival differences were tested by the Kaplan-Meier method. The difference was considered to be significant when $p<0.05$.

Results

Upregulation of miR-572 was Identified in WT Tissues

First, the expression of miR-572 was detected in WT tissues and cell lines. MiR-572 expression was significantly higher in WT tissues than that of normal tissues (Figure 1A). Similarly, upregulation of miR-572 was also observed in HFWT and 17-94 cells compared to the control (Figure 1B). Moreover, the correlation between the clinical characteristics of WT patients and miR-572 expression was investigated. The results showed that miR-572 expression was markedly related to the histological type ($p=0.03$), the lymphatic metastasis ($p=0.04$) and the NWTS-5 stage ($p=0.01$), as shown in Table I. In addition, WT patients with high miR-572 expression had shorter overall survival rate ($p=0.0002$, Figure 1C). These results suggested that the dysregulation of miR-572 might be involved in the tumorigenesis of WT, and aberrant miR-572 expression could be considered as a potential biomarker for the prognosis of WT.

Upregulation of miR-572 Promoted Cell Metastasis and EMT in WT

Next, miR-572 mimics or inhibitor was transfected into 17-94 cells to explore its function

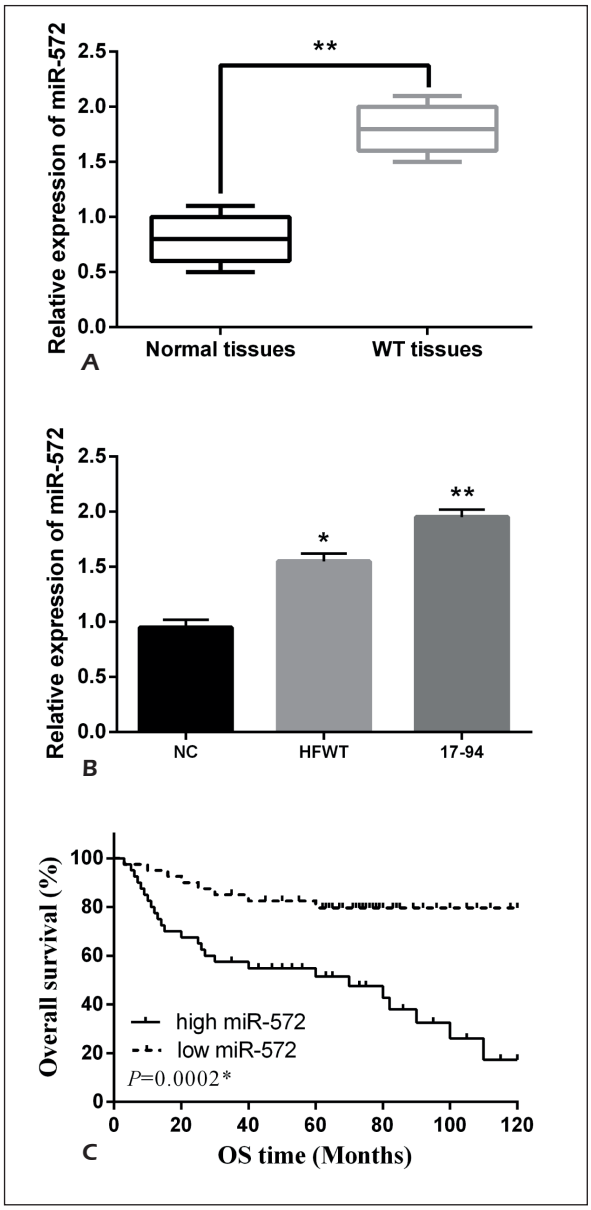

Figure 1. Upregulation of miR-572 was identified in WT tissues. **A**, MiR-572 expressions in WT tissues and normal tissues were detected via qRT-PCR. **B**, The miR-572 expression in HFWT and 17-94 cells. C, Upregulation of miR-572 showed shorter overall survival in WT patients. *$p<0.05$, **$p<0.01$.

in WT. The expression level of miR-572 was enhanced by miR-572 mimics and reduced by miR-572 inhibitor (Figure 2A). Functionally, the upregulation of miR-572 remarkably promoted cell migration, and the migration of 17-94 cells was suppressed by knockdown of miR-572 (Figure 2B). Besides, the invasion of 17-94 cells transfected with miR-572 mimics or inhibitor was similar to the tendency of cell migration (Figure 2C). In order

Table I. Correlation between the clinicopathologic characteristics and miR-572 in Wilms' tumors patients.

Characteristics	Number of Cases (n=61)	miR-572		*p*-value
		High (n=43)	Low (n=18)	
Age (Months)				2.24
≥ 24	25	15	10	
< 24	36	28	8	
Gender				1.07
Male	38	25	13	
Female	23	18	5	
Histological type				0.03*
Favorable (FH)	45	32	13	
Unfavorable (UH)	16	11	5	
Lymphatic metastasis				0.04*
Absent	43	30	13	
Present	18	13	5	
NWTS-5 stage				0.01*
I + II	47	33	14	
III + IV	14	10	4	

Statistical analyses were performed by the χ^2 test. *p<0.05 was considered significant.

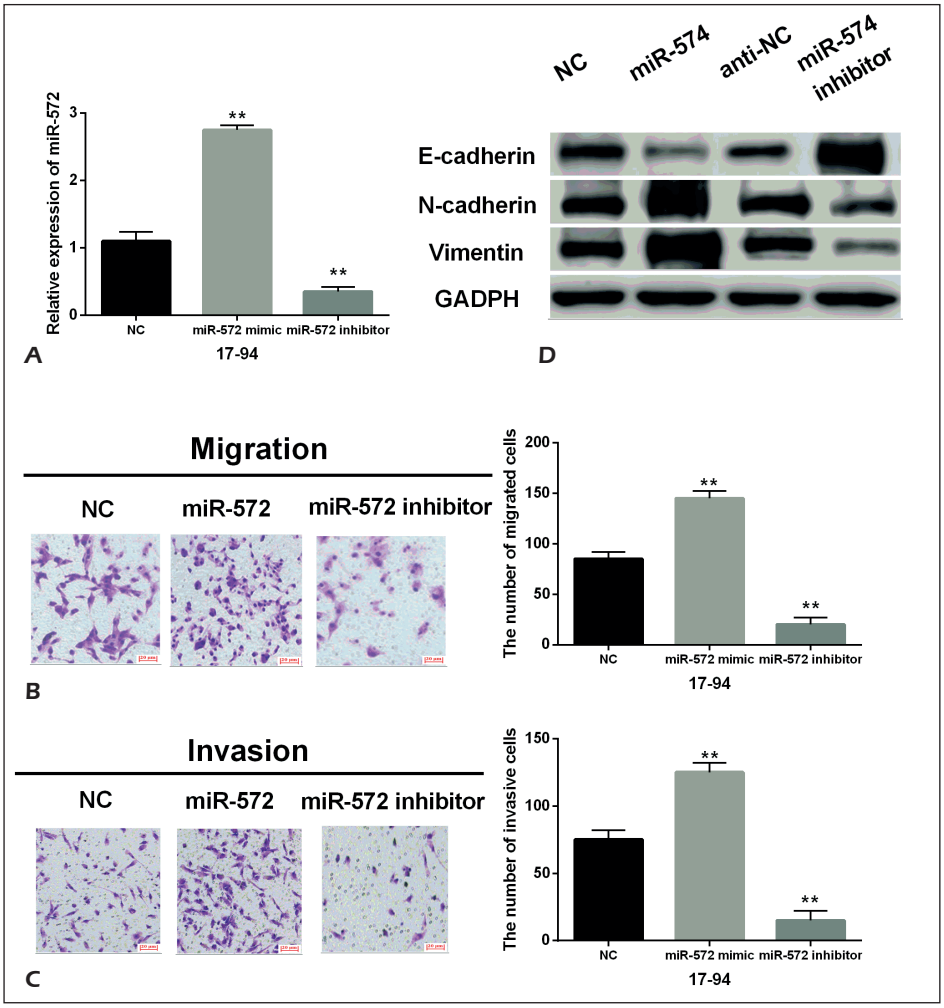

Figure 2. Upregulation of miR-572 promoted cell metastasis and EMT in WT. **A**, MiR-572 expression was observed in 17-94 cells transfected with miR-572 mimics or inhibitor. **B-C**, The cell migration and invasion were observed in transfected 17-94 cells. **D**, Western blot analysis of E-cadherin, N-cadherin and Vimentin in transfected 17-94 cells. **p<0.01.

to confirm the effect of miR-572 on cell metastasis, EMT progress of 17-94 cells was also investigated. The Western blot assay showed that upregulation of miR-572 inhibited E-cadherin expression while promoted the N-cadherin and Vimentin expressions. Inversely, knockdown of miR-572 had the opposite effect on their expressions in 17-94 cells (Figure 2D). Collectively, miR-572 promoted the cell metastasis by activating EMT in WT.

CDH1 was a Direct Target of miR-572

Further, Targetscan (http://www.targetscan.org) was used for target genes prediction to explain the regulatory mechanism underlying miR-572 in cell metastasis. CDH1 was then selected as a target gene of miR-572 (Figure 3A). To verify whether miR-572 regulated CDH1 through the binding sites, dual-luciferase reporter assay was then performed. We found that miR-572 reduced the luciferase activity of CDH1-wt group, but had no effect on CDH1-mut group (Figure 3B). Furthermore, a negative correlation between miR-572 and CDH1 was identified in WT tissues (p<0.0001, R^2=0.6129; Figure 3C). Next, the expression of CDH1 was measured in 17-94 cells with miR-572 mimics and inhibitor. The CDH1 expression was reduced by miR-572 mimics, but enhanced by miR-572 inhibitor (Figure 3D, 3E). To sum up, miR-572 directly targeted CDH1 and negatively regulated CDH1 expression in WT.

The Knockdown of CDH1 Promoted Cell Metastasis and EMT in WT

Subsequently, we found that CDH1 was downregulated in WT tissues and cell lines (Figure 4A, 4B). In order to explore the effect of CDH1 on cell metastasis, CDH1 siRNA was transfected into 17-94 cells. CDH1 siRNA significantly decreased the expression of CDH1 in 17-94 cells (Figure 4C). Functionally, the knockdown of CDH1 was found to promote the migration and invasion of 17-94 cells (Figure 4D, 4E). Moreover, the effect of CDH1 on EMT was also analyzed in 17-94 cells. The Western blot showed that CDH1 silence enhanced the N-cadherin and Vimentin expressions and declined the E-cadherin expression (Figure 4F). Therefore, CDH1 was a tumor suppressive

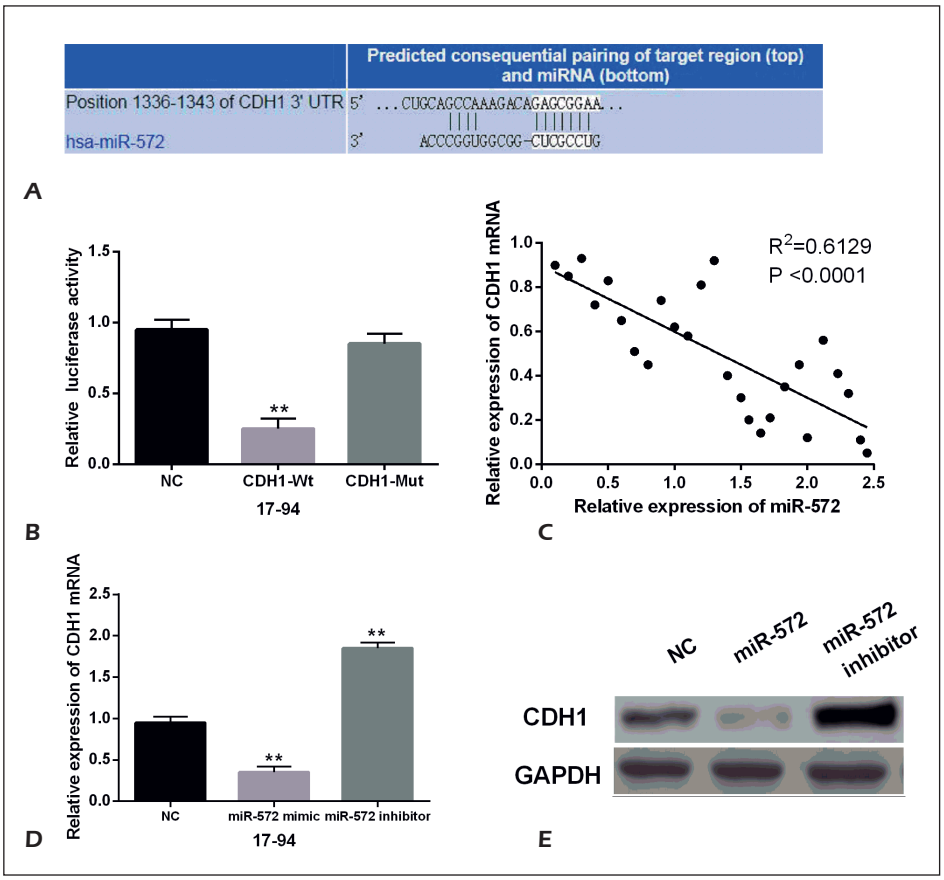

Figure 3. CDH1 was a direct target of miR-572. **A**, CDH1 has binding sites with MiR-572. **B**, Luciferase reporter assay. **C**, The negative association between miR-572 and CDH1 was detected in WT tissues. **D-E**, The CDH1 expression was analyzed in 17-94 cells transfected with miR-572 mimics or inhibitor **p<0.01.

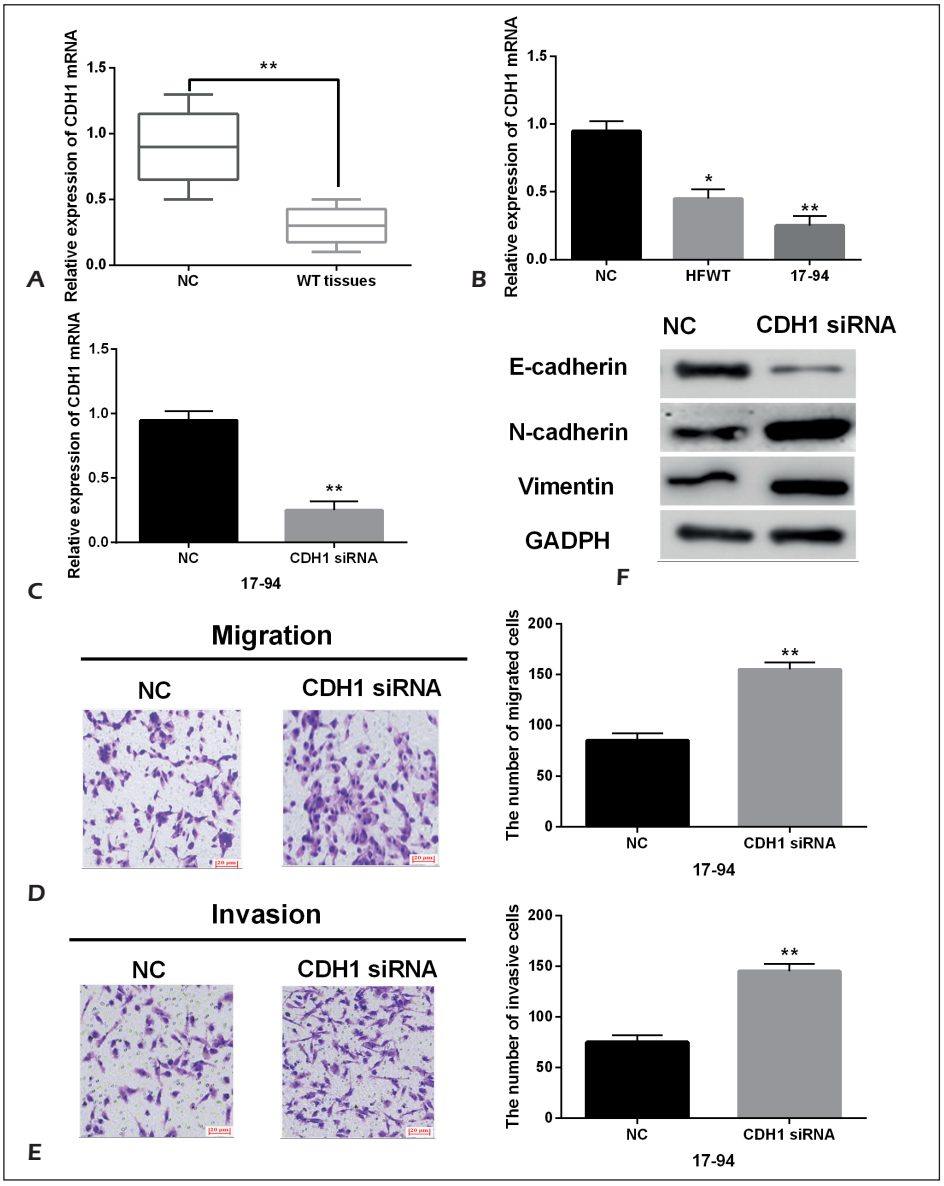

Figure 4. The knockdown of CDH1 promoted cell metastasis and EMT in WT. **A**, The CDH1 expression was identified in WT tissues. **B**, The CDH1 expression in HFWT and 17-94 cells. **C**, The CDH1 expression was observed in 17-94 cells transfected with CDH1 siRNA. **D-E**, Cell migration and invasion analysis of 17-94 cells transfected with CDH1 siRNA was detected by Transwell assay. **F**, The expressions of EMT markers were identified in 17-94 cells transfected with CDH1 siRNA. *p<0.05, **p<0.01.

gene in WT through inhibiting cell metastasis.

Overexpression of CDH1 Weakened the Effect of miR-572 in WT

To further verify whether miR-572 regulated the metastasis of WT cells through targeting CDH1, miR-572 mimic and CDH1 vector were transfected into 17-94 cells. As predicted, overexpression of CDH1 restored the decreased CDH1 expression induced by miR-572 mimics (Figure 5A, 5B). Moreover, the enhancement of cell migration and invasion induced by miR-572 mimics was weakened by the transfection of CDH1 vector in 17-94 cells (Figure 5C, 5D). Taken together, the carcinogenic effect of miR-572 on cell metastasis was impaired by overexpression of CDH1, indicating that miR-572 promoted cell metastasis by regulating CDH1 in WT.

Discussion

Although WT has become one of the high-risk diseases in children resulting in death, there are few studies on its pathogenesis. Moreover, only a few miRNAs associated with WT have been reported. For example, upregulation of miR-21 was identified in WT with aggressive behavior[20]. Cui et al[21] proposed that abnormal miR-21 was related to the lymphatic metastasis, the histopathological tumor type and the late clinical stage as well as the prognosis of WT patients. Similarly, the upregulation of miR-572 was identified in WT and was associated with worse outcome in WT pa-

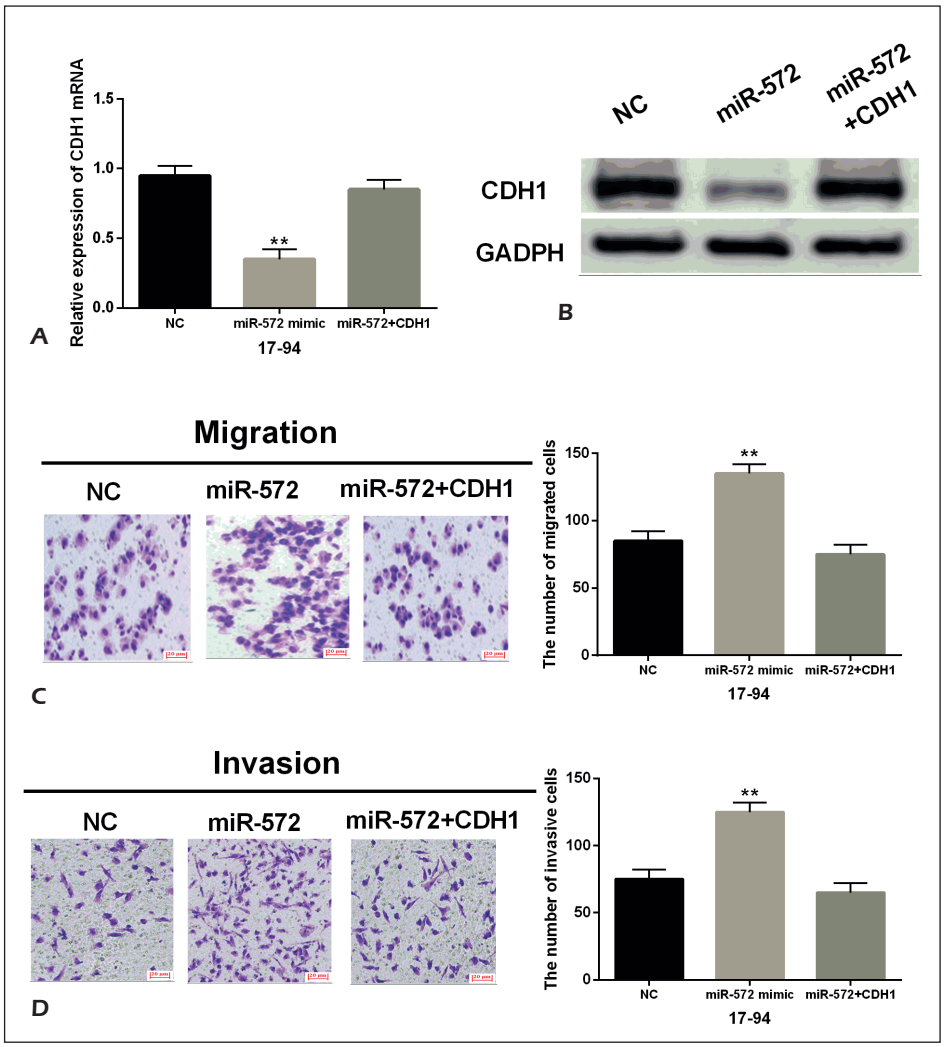

Figure 5. Overexpression of CDH1 weakened the effect of miR-572 on WT. **A-B**, CDH1 expression was measured in 17-94 cells transfected with CDH1 vector and miR-572 mimic. **C-D**, The cell migration and invasion in 17-94 cells transfected with with CDH1 vector and miR-572 mimic was examined. $^{**}p<0.01$.

tients. Functionally, increased miR-572 promoted the cell metastasis and EMT in WT. To the best of our knowledge, this is the first demonstration that miR-572 played a carcinogenic role in WT by regulating cell metastasis. Consistent with our findings, miR-572 could be used as a carcinogenic factor in the development of certain human cancers. It has been demonstrated that miR-572 expression was increased in neuroblastoma cells[22]. Upregulation of miR-572 was also found to transcriptionally suppress SOCS1 and contribute to human ovarian cancer progression[23]. In this study, miR-572 regulated the development of WT by promoting cell migration, invasion and EMT. Consistently, overexpression of miR-572 could promote the proliferation and invasion of hepatocellular carcinoma[24]. Moreover, miR-572 has been identified as a potential diagnostic tool for early-stage renal cell carcinoma[25]. All these findings implied that carcinogenic miR-572 is involved in the pathogenesis of WT. Furthermore, miR-572 directly targeted CDH1 and negatively regulated CDH1 expression in WT. Moreover, downregulation of CDH1 exhibited a suppressive effect on cell metastasis and EMT. Previous studies have shown that CDH1 was a direct target gene of several miRNAs, such as miR-9, miR-23a and miR-199a[26-28]. Same as the effect of CDH1 in WT, the knockdown of CDH1 had been reported to promote cell metastasis and EMT in neuroblastoma[29]. Lu *et al*[30] revealed that overexpression of CDH1 weakened the effect of miR-544a on cell migration and invasion in breast cancer, which was also consistent with our results. In addition, it was demonstrated that miR-217 was involved in the carcinogenesis of gastric cancer by down-regulating CDH1 expression[31]. Especially, miR-720 regulated CDH1 to promote EMT and metastasis in renal cell carcinoma[32]. Based on these results, we inferred that miR-572 functioned as tumor promoter through targeting CDH1 to

promote metastasis in WT.

Conclusions

We showed that miR-572 was up-regulated in WT. Furthermore, upregulation of miR-572 inhibited CDH1 expression and induced the metastasis of WT cells. We concluded that miR-572 targeted CDH1 to promote cell metastasis in WT by suppressing EMT. Together, these findings suggested that miR-572 might be a therapeutic target for WT in the future.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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